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(54) Title: FLUORESCENT PROTEINS FROM NON–BIOLUMINESCENT SPECIES OF CLASS ANTHOZOA, GENES ENCODING SUCH PROTEINS AND USES THEREOF

(57) Abstract

The present invention is directed to novel fluorescent proteins from non–bioluminescent organisms from the Class Anthozoa. Also disclosed are methods of identifying nucleic acid sequence encoding the fluorescent proteins and further analyzing the proteins.

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FLUORESCENT PROTEINS FROM NON-BIOLUMINESCENT SPECIES OF CLASS ANTHOZOA, GENES ENCODING SUCH PROTEINS AND USES THEREOF

BACKGROUND OF THE INVENTION

Field of the Invention

This invention relates to the field of molecular biology. More specifically, this invention relates to novel fluorescent proteins, methods of identifying the DNA sequences encoding the proteins and uses thereof.

Description of the Related Art

Fluorescence labeling is a particularly useful tool for marking a protein, cell, or organism of interest. Traditionally, a protein of interest is purified, then covalently conjugated to a fluorophore derivative. For *in vivo* studies, the protein-dye complex is then inserted into cells of interest using micropipetting or a method of reversible permeabilization. The dye attachment and insertion steps, however, make the process laborious and difficult to control. An alternative method of labeling proteins of interest is to concatenate or fuse the gene expressing the protein of interest to a gene expressing a marker, then express the fusion product. Typical markers for this method of protein labeling include β-galactosidase, firefly luciferase

and bacterial luciferase. These markers, however, require exogenous substrates or cofactors and are therefore of limited use for *in vivo* studies.

A marker that does not require an exogenous cofactor or substrate is the green fluorescent protein (GFP) of the jellyfish *Aequorea victoria*, a protein with an excitation maximum at 395 nm, a second excitation peak at 475 nm and an emission maximum at 510 nm. GFP is a 238-amino acid protein, with amino acids 65-67 involved in the formation of the chromophore.

Uses of GFP for the study of gene expression and protein localization are discussed in detail by Chalfie et al. in *Science* 263 (1994), 802-805, and Heim et al. in *Proc. Nat. Acad. Sci.* 91 (1994), 12501-12504. Additionally, Rizzuto et al. in *Curr. Biology* 5 (1995), 635-642, discuss the use of wild-type GFP as a tool for visualizing subcellular organelles in cells, while Kaether and Gerdes in *Febs Letters* 369 (1995), 267-271, report the visualization of protein transport along the secretory pathway using wild-type GFP. The expression of GFP in plant cells is discussed by Hu and Cheng in *Febs Letters* 369 (1995), 331-334, while GFP expression in *Drosophila* embryos is described by Davis et al. in *Dev. Biology* 170 (1995), 726-729.

Crystallographic structures of wild-type GFP and the mutant GFP S65T reveal that the GFP tertiary structure resembles a barrel (Ormö et al., *Science* 273 (1996), 1392-1395; Yang, et al., *Nature Biotechnol* 14 (1996), 1246-1251). The barrel consists of beta sheets in a compact structure, where, in the center, an alpha helix containing the chromophore is shielded by the barrel. The compact structure makes GFP very stable under diverse and/or harsh conditions such as protease treatment, making GFP an extremely useful reporter in

general. However, the stability of GFP makes it sub-optimal for determining short-term or repetitive events.

A great deal of research is being performed to improve the properties of GFP and to produce GFP reagents useful and optimized for a variety of research purposes. New versions of GFP have been developed, such as a "humanized" GFP DNA, the protein product of which has increased synthesis in mammalian cells (Haas, et al., *Current Biology* 6 (1996), 315-324; Yang, et al., *Nucleic Acids Research* 24 (1996), 4592-4593). One such humanized protein is "enhanced green fluorescent protein" (EGFP). Other mutations to GFP have resulted in blue-, cyan- and yellow-green light emitting versions. Despite the great utility of GFP, however, other fluorescent proteins with properties similar to or different from GFP would be useful in the art. Novel fluorescent proteins result in possible new colors, or produce pH-dependent fluorescence. Other benefits of novel fluorescent proteins include fluorescence resonance energy transfer (FRET) possibilities based on new spectra and better suitability for larger excitation.

The prior art is deficient in novel fluorescent proteins wherein the DNA coding sequences are known. The present invention fulfills this long-standing need in the art.

SUMMARY OF THE INVENTION

The present invention is directed to an isolated and purified fluorescent protein selected from the group consisting of amFP486, cFP484, zFP506, zFP538, dsFP483, drFP583, asFP600, dgFP512 and dmFP592.

In one embodiment of the present invention, there is provided a method of identifying a DNA sequence encoding a fluorescent protein comprising the step of screening for an existence of a nucleic acid sequence in a sample, wherein the nucleic acid sequence encodes a peptide having a sequence selected from the group consisting of SEQ ID Nos. 3, 5, 8, 11, 12 and 14. The existence of the nucleic acid sequence identifies the DNA sequence encoding the fluorescent protein.

In another embodiment of the present invention, there is provided a method of identifying a DNA sequence encoding a fluorescent protein comprising the step of screening for an existence of a nucleic acid sequence in a sample, wherein the nucleic acid sequence hybridizes to a primer selected from the group consisting of SEQ ID Nos. 4, 6, 7, 9, 10, 13, 15 and 16. The existence of the nucleic acid sequence identifies the DNA sequence encoding the fluorescent protein.

In still another embodiment of the present invention, there is provided a method of analyzing a fluorescent protein in a cell, comprising the steps of expressing a nucleic acid sequence encoding a fluorescent protein having an amino acid sequence selected from the group consisting of SEQ ID Nos. 55-63 in the cell; and measuring a fluorescence signal from the protein. This method further comprises a step of sorting the cell according to the signal. Preferably, the cell is sorted by fluorescence activated cell sorting. Still preferably, the nucleic acid sequence comprises a gene of interest encoding a protein of interest fused to the fluorescent protein, wherein the protein of interest is distinct from the fluorescent protein. The detected fluorescence signal indicates the presence of the gene of interest and further the protein of interest in the cell. By identifying an

intracellular location of the fluorescent protein, an intracellular location of the protein of interest is also identified.

Other and further aspects, features, and advantages of the present invention will be apparent from the following description of the presently preferred embodiments of the invention given for the purpose of disclosure.

BRIEF DESCRIPTION OF THE DRAWINGS

Figure 1 shows the modified strategy of 3'-RACE used to isolate the target fragments. Sequences of the oligonucleotides used are shown in Table 2. Dp1 and Dp2 are the degenerate primers used in the first and second PCR, respectively (see Tables 3 and 4 for the sequences of degenerate primers).

Figure 2A shows multiple alignment of novel fluorescent proteins. The numbering is based on *Aequorea victoria* green fluorescent protein (GFP). Two proteins from *Zoanthus* and four from *Discosoma* are compared between each other: residues identical to the corresponding ones in the first protein of the series are represented by dashes. Introduced gaps are represented by dots. In the sequence of *A. victoria* GFP, the stretches forming beta-sheets are underlined; the residues whose side chains form the interior of the beta-can are shaded (according to Yang et al., *Nature Biotechnol.* 14, 1246–1251 (1996). **Figure 2B** shows the N-terminal part of cFP484, which has no homology with the other proteins. The putative signal peptide is underlined.

Figure 3 shows the excitation and emission spectrum of the novel fluorescent protein from *Anemonia majano*, amFP486.

Figure 4 shows the excitation and emission spectrum of the novel fluorescent protein from *Clavularia*, cFP484.

Figure 5 shows the excitation and emission spectrum of the novel fluorescent protein from *Zoanthus*, zFP506.

Figure 6 shows the excitation and emission spectrum of the novel fluorescent protein from *Zoanthus*, zFP538.

Figure 7 shows the excitation and emission spectrum of the novel fluorescent protein from *Discosoma striata*, dsFP483.

Figure 8 shows the excitation and emission spectrum of the novel fluorescent protein from *Discosoma*, drFP583.

Figure 9 shows the excitation and emission spectrum of the novel fluorescent protein from *Anemonia sulcata*, asFP600.

Figure 10 shows the excitation and emission spectrum of the novel fluorescent protein from *Discosoma*, dgFP512.

Figure 11 shows the excitation and emission spectrum of the novel fluorescent protein from *Discosoma*, dmFP592.

DETAILED DESCRIPTION OF THE INVENTION

As used herein, the term "GFP" refers to the basic green fluorescent protein from *Aequorea victoria*, including prior art versions of GFP engineered to provide greater fluorescence or fluoresce in different colors. The sequence of *Aequorea victoria* GFP (SEQ ID No. 54) has been disclosed in Prasher et al., *Gene* 111 (1992), 229-33.

As used herein, the term "EGFP" refers to mutant variant of GFP having two amino acid substitutions: F64L and S65T (Heim et al., Nature 373 (1995), 663-664). The term "humanized" refers to changes made to the GFP nucleic acid sequence to optimize the codons for

expression of the protein in human cells (Yang et al., *Nucleic Acids Research* 24 (1996), 4592-4593).

In accordance with the present invention there may be employed conventional molecular biology, microbiology, and recombinant DNA techniques within the skill of the art. Such techniques are explained fully in the literature. See, e.g., Maniatis, Fritsch & Sambrook, "Molecular Cloning: A Laboratory Manual (1982); "DNA Cloning: A Practical Approach," Volumes I and II (D.N. Glover ed. 1985); "Oligonucleotide Synthesis" (M.J. Gait ed. 1984); "Nucleic Acid Hybridization" (B.D. Hames & S.J. Higgins eds. (1985)); "Transcription and Translation" (B.D. Hames & S.J. Higgins eds. (1984)); "Animal Cell Culture" (R.I. Freshney, ed. (1986)); "Immobilized Cells and Enzymes" (IRL Press, (1986)); B. Perbal, "A Practical Guide To Molecular Cloning" (1984).

A "vector" is a replicon, such as plasmid, phage or cosmid, to which another DNA segment may be attached so as to bring about the replication of the attached segment.

A "DNA molecule" refers to the polymeric form of deoxyribonucleotides (adenine, guanine, thymine, or cytosine) in either single stranded form or a double-stranded helix. This term refers only to the primary and secondary structure of the molecule, and does not limit it to any particular tertiary forms. Thus, this term includes double-stranded DNA found, inter alia, in linear DNA molecules (e.g., restriction fragments), viruses, plasmids, and chromosomes.

A DNA "coding sequence" is a DNA sequence which is transcribed and translated into a polypeptide in vivo when placed under the control of appropriate regulatory sequences. The boundaries of the coding sequence are determined by a start codon at the 5' (amino) terminus and a translation stop codon at the 3'

(carboxyl) terminus. A coding sequence can include, but is not limited to, prokaryotic sequences, cDNA from eukaryotic mRNA, genomic DNA sequences from eukaryotic (e.g., mammalian) DNA, and synthetic DNA sequences. A polyadenylation signal and transcription termination sequence may be located 3' to the coding sequence.

As used herein, the term "hybridization" refers to the process of association of two nucleic acid strands to form an antiparallel duplex stabilized by means of hydrogen bonding between residues of the opposite nucleic acid strands.

The term "oligonucleotide" refers to a short (under 100 bases in length) nucleic acid molecule.

"DNA regulatory sequences", as used herein, are transcriptional and translational control sequences, such as promoters, enhancers, polyadenylation signals, terminators, and the like, that provide for and/or regulate expression of a coding sequence in a host cell.

A "promoter sequence" is a DNA regulatory region capable of binding RNA polymerase in a cell and initiating transcription of a downstream (3' direction) coding sequence. For purposes of defining the present invention, the promoter sequence is bounded at its 3' terminus by the transcription initiation site and extends upstream (5' direction) to include the minimum number of bases or elements necessary to initiate transcription at levels detectable above background. Within the promoter sequence will be found a transcription initiation site, as well as protein binding domains responsible for the binding of RNA polymerase. Eukaryotic promoters will often, but not always, contain "TATA" boxes and "CAT" boxes. Various promoters, including inducible promoters, may be used to drive the various vectors of the present invention.

As used herein, the terms "restriction endonucleases" and "restriction enzymes" refer to bacterial enzymes, each of which cut double-stranded DNA at or near a specific nucleotide sequence.

A cell has been "transformed" or "transfected" by exogenous or heterologous DNA when such DNA has been introduced inside the cell. The transforming DNA may or may not be integrated (covalently linked) into the genome of the cell. In prokaryotes, yeast, and mammalian cells for example, the transforming DNA may be maintained on an episomal element such as a plasmid. With respect to eukaryotic cells, a stably transformed cell is one in which the transforming DNA has become integrated into a chromosome so that it is inherited by daughter cells through chromosome replication. This stability is demonstrated by the ability of the eukaryotic cell to establish cell lines or clones comprised of a population of daughter cells containing the transforming DNA. A "clone" is a population of cells derived from a single cell or common ancestor by mitosis. A "cell line" is a clone of a primary cell that is capable of stable growth *in vitro* for many generations.

A "heterologous" region of the DNA construct is an identifiable segment of DNA within a larger DNA molecule that is not found in association with the larger molecule in nature. Thus, when the heterologous region encodes a mammalian gene, the gene will usually be flanked by DNA that does not flank the mammalian genomic DNA in the genome of the source organism. In another example, heterologous DNA includes coding sequence in a construct where portions of genes from two different sources have been brought together so as to produce a fusion protein product. Allelic variations or naturally-occurring mutational events do not give rise to a heterologous region of DNA as defined herein.

As used herein, the term "reporter gene" refers to a coding sequence attached to heterologous promoter or enhancer elements and whose product may be assayed easily and quantifiably when the construct is introduced into tissues or cells.

The amino acids described herein are preferred to be in the "L" isomeric form. The amino acid sequences are given in one-letter code (A: alanine; C: cysteine; D: aspartic acid; E: gluetamic acid; F: phenylalanine; G: glycine; H: histidine; I: isoleucine; K: lysine; L: leucine; M: metionine; N: asparagine; P: proline; Q: gluetamine; R: arginine; S: serine; T: threonine; V: valine; W: tryptophane; Y: tyrosine; X: any residue). NH_2 refers to the free amino group present at the amino terminus of a polypeptide. COOH refers to the free carboxy group present at the carboxy terminus of a polypeptide. In keeping with standard polypeptide nomenclature, *J Biol. Chem.*, 243 (1969), 3552-59 is used.

The present invention is directed to an isolated and purified fluorescent protein selected from the group consisting of amFP486, cFP484, zFP506, zFP538, dsFP483, drFP583, asFP600, dgFP512 and dmFP592.

In one embodiment of the present invention, there is provided a method of identifying a DNA sequence encoding a fluorescent protein comprising the step of screening for an existence of a nucleic acid sequence in a sample, wherein the nucleic acid sequence encodes a peptide having a sequence selected from the group consisting of SEQ ID Nos. 3, 5, 8, 11, 12 and 14. The existence of the nucleic acid sequence identifies the DNA sequence encoding the fluorescent protein.

In another embodiment of the present invention, there is provided a method of identifying a DNA sequence encoding a

fluorescent protein comprising the step of screening for an existence of a nucleic acid sequence in a sample, wherein the nucleic acid sequence hybridizes to a primer selected from the group consisting of SEQ ID Nos. 4, 6, 7, 9, 10, 13, 15 and 16. The existence of the nucleic acid sequence identifies the DNA sequence encoding the fluorescent protein.

In still another embodiment of the present invention, there is provided a method of analyzing a fluorescent protein in a cell, comprising the steps of expressing a nucleic acid sequence encoding a fluorescent protein having an amino acid sequence selected from the group consisting of SEQ ID Nos. 55-63 in the cell; and measuring a fluorescence signal from the protein. This method further comprises a step of sorting the cell according to the signal. Preferably, the cell is sorted by fluorescence activated cell sorting. Still preferably, the nucleic acid sequence comprises a gene of interest encoding a protein of interest fused to the fluorescent protein, wherein the protein of interest is distinct from the fluorescent protein. The detected fluorescence signal indicates the presence of the gene of interest and further the protein of interest in the cell. By identifying an intracellular location of the fluorescent protein, an intracellular location of the protein of interest is also identified.

The following examples are given for the purpose of illustrating various embodiments of the invention and are not meant to limit the present invention in any fashion.

EXAMPLE 1

Biological Material

Novel fluorescent proteins were identified from several genera of Anthozoa which do not exhibit any bioluminescence but have fluorescent color as observed under usual white light or ultraviolet light. Six species were chosen (see Table 1).

TABLE 1

Anthozoa Species Used in This Study

Species	Area of Origination	Fluorescent Color
Anemonia majano	Western Pacific	bright green tentacle tips
Clavularia sp.	Western Pacific	bright green tentacles and oral disk
Zoanthus sp.	Western Pacific	green-yellow tentacles and oral disk
Discosoma sp. "red"	Western Pacific	orange-red spots oral disk
Discosoma striata	Western Pacific	blue-green stripes on oral disk
Discosoma sp. "magenta"	Western Pacific	faintly purple oral disk
Discosoma sp.	Western Pacific	green spots on oral disk

"green"		
Anemonia sulcata	Mediterranean	purple tentacle tips

EXAMPLE 2

cDNA Preparation

Total RNA was isolated from the species of interest according to the protocol of Chomczynski and Sacchi (Chomczynski P., et al., *Anal. Biochem.* 162 (1987), 156-159). First-strand cDNA was synthetized starting with 1-3 µg of total RNA using SMART PCR cDNA synthesis kit (CLONTECH) according to the provided protocol with the only alteration being that the "cDNA synthesis primer" provided in the kit was replaced by the primer TN3 (5'- $CGCAGTCGACCG(T)_{13}$, SEQ ID No. 1) (Table 2). Amplified cDNA samples were then prepared as described in the protocol provided except the two primers used for PCR were the TS primer (5'-AAGCAGTGGTATCAACGCAGAGT, SEQ ID No. 2) (Table 2) and the TN3 primer (Table 2), both in 0.1 µM concentration. Twenty to twenty-five PCR cycles were performed to amplify a cDNA sample. The amplified cDNA was diluted 20-fold in water and 1 µl of this dilution was used in subsequent procedures.

TABLE 2

Oligos Used in cDNA Synthesis and RACE

TN3: 5'-CGCAGTCGACCG$(T)_{13}$
(SEQ ID No. 1)

T7-TN3: 5'-GTAATACGACTCACTATAGGGCCGCAGTCGACCG$(T)_{13}$
(SEQ ID No. 17)

TS-primer: 5'-AAGCAGTGGTATCAACGCAGAGT
(SEQ ID No. 2)

T7-TS:
5'-GTAATACGACTCACTATAGGGCAAGCAGTGGTATCAACGCAGAGT
(SEQ ID No. 18)

T7: 5'-GTAATACGACTCACTATAGGGC
(SEQ ID No. 19)

TS-oligo 5'-AAGCAGTGGTATCAACGCAGAGTACGCrGrGrG
(SEQ ID No. 53)

EXAMPLE 3

Oligo Design

To isolate fragments of novel fluorescent protein cDNAs, PCR using degenerate primers was performed. Degenerate primers were designed to match the sequence of the mRNAs in regions that were predicted to be the most invariant in the family of fluorescent proteins. Four such stretches were chosen (Table 3) and variants of degenerate primers were designed. All such primers were directed to the 3'-end of mRNA. All oligos were gel-purified before use. Table 2 shows the oligos used in cDNA synthesis and RACE.

TABLE 3

Key Amino Acid Stretches and Corresponding Degenerate Primers Used for Isolation of Fluorescent Proteins

Stretch Position according to A. victoria GFP (7)	Amino Acid Sequence of the Key Stretch	Degenerated Primer Name and Sequence
20-25	GXVNGH (SEQ ID No. 3)	NGH: 5'- GA(C,T) GGC TGC GT(A,T,G,C) AA(T,C) GG(A,T,G) CA (SEQ ID No. 4)
31-35	GEGEG (SEQ ID No. 5)	GEGa: 5'- GTT ACA GGT GA(A,G) GG(A,C) GA(A,G) GG (SEQ ID No. 6) GEGb: 5'- GTT ACA GGT GA(A,G) GG(T,G) GA(A,G) GG (SEQ ID No. 7)
	GEGNG (SEQ ID No. 8)	GNGa: 5'- GTT ACA GGT GA(A,G) GG(A,C) AA(C,T) GG (SEQ ID No. 9) GNGb: 5'- GTT ACA GGT GA(A,G) GG(T,G) AA(C,T) GG (SEQ ID No. 10)
127-131	GMNFP (SEQ ID No. 11) GVNFP (SEQ ID No. 12)	NFP: 5' TTC CA(C,T) GGT (G,A)TG AA(C,T) TT(C,T) CC (SEQ ID NO. 13)
134-137	GPVM (SEQ ID No. 14)	PVMa: 5' CCT GCC (G,A)A(C,T) GGT CC(A,T,G,C) GT(A,C) ATG (SEQ ID NO. 15) PVMb: 5' CCT GCC (G,A)A(C,T) GGT CC(A,T,G,C) GT(G,T) ATG (SEQ ID NO. 16)

EXAMPLE 4

Isolation of 3'-cDNA Fragments of nFPs

The modified strategy of 3'-RACE was used to isolate the target fragments (see Figure 1). The RACE strategy involved two consecutive PCR steps. The first PCR step involved a first degenerate primer (Table 4) and the T7-TN3 primer (SEQ ID No. 17) which has a 3' portion identical to the TN3 primer used for cDNA synthesis (for sequence of T7-TN3, Table 2). The reason for substituting the longer T7-TN3 primer in this PCR step was that background amplification which occurred when using the shorter TN3 primer was suppressed effectively, particularly when the T7-TN3 primer was used at a low concentration (0.1 _M) (Frohman et al., (1998) *PNAS USA*, 85, 8998-9002). The second PCR step involved the TN3 primer (SEQ ID No. 1, Table 2) and a second degenerate primer (Table 4).

TABLE 4

Combinations of Degenerate Primers for First and Second PCR Resulting in Specific Amplification of 3'-Fragments of nFP cDNA

Species	First Degenerate Primer	Second Degenerate Primer
Anemonia majano	NGH (SEQ ID No. 4)	GNGb (SEQ ID No. 10)
Clavularia sp.	NGH (SEQ ID No. 4)	GEGa (SEQ ID No. 6)
Zoanthus sp.	NGH (SEQ ID No. 4)	GEGa (SEQ ID No. 6)
Discosoma sp. "red"	NGH (SEQ ID No. 4)	GEGa (SEQ ID No. 6), NFP (SEQ ID No. 13) or PVMb (SEQ ID No. 16)
Discosoma striata	NGH (SEQ ID No. 4)	NFP (SEQ ID No. 13)
Anemonia sulcata	NGH (SEQ ID No. 4)	GEGa (SEQ ID No. 6) or NFP (SEQ ID No. 13)

The first PCR reaction was performed as follows: 1 µl of 20-fold dilution of the amplified cDNA sample was added into the reaction mixture containing 1X Advantage KlenTaq Polymerase Mix with provided buffer (CLONTECH), 200 µM dNTPs, 0.3 µM of first degenerate

primer (Table 4) and 0.1 μM of T7-TN3 (SEQ ID No. 17) primer in a total volume of 20 μl. The cycling profile was (Hybaid OmniGene Thermocycler, tube control mode): 1 cycle for 95°C, 10 sec.; 55°C, 1 min.; 72°C, 40 sec; 24 cycles for 95°C, 10 sec.; 62°C, 30 sec.; 72°C, 40 sec. The reaction was then diluted 20-fold in water and 1 μl of this dilution was added to a second PCR reaction, which contained 1X Advantage KlenTaq Polymerase Mix with the buffer provided by the manufacturer (CLONTECH), 200 μM dNTPs, 0.3 μM of the second degenerate primer (Table 4) and 0.1 μM of TN3 primer. The cycling profile was (Hybaid OmniGene Thermocycler, tube control mode): 1 cycle for 95°C, 10 sec.; 55°C (for GEG/GNG or PVM) or 52°C (for NFP), 1 min.; 72°C, 40 sec; 13 cycles for 95°C, 10sec.; 62°C (for GEG/GNG or PVM) or 58°C (for NFP), 30 sec.; 72°C, 40 sec. The product of PCR was cloned into PCR-Script vector (Stratagene) according to the manufacturer's protocol.

Different combinations of degenerate primers were tried in the first and second PCR reactions on the DNA from each species until a combination of primers was found that resulted in specific amplification--meaning that a pronounced band of expected size (about 650-800 bp for NGH and GEG/GNG and 350-500 bp for NFP and PVM--sometimes accompanied by a few minor bands) was detected on agarose gel after two PCR reactions. The primer combinations of choice for different species of the Class Anthozoa are listed in Table 4. Some other primer combinations also resulted in amplification of fragments of correct size, but the sequence of these fragments showed no homology to the other fluorescent proteins identified or to *Aequorea victoria* GFP.

EXAMPLE 5

Obtaining Full-Length cDNA Copies

Upon sequencing the obtained 3'-fragments of novel fluorescent protein cDNAs, two nested 5'-directed primers were synthesized for cDNA (Table 5), and the 5' ends of the cDNAs were then amplified using two consecutive PCRs. In the next PCR reaction, the novel approach of "step-out PCR" was used to suppress background amplification. The step-out reaction mixture contained 1x Advantage KlenTaq Polymerase Mix using buffer provided by the manufacturer (CLONTECH), 200 μM dNTPs, 0.2 μM of the first gene-specific primer (see Table 5), 0.02 μM of the T7-TS primer (SEQ ID No. 18), 0.1 μM of T7 primer (SEQ ID No. 19) and 1 μl of the 20-fold dilution of the amplified cDNA sample in a total volume of 20 μl. The cycling profile was (Hybaid OmniGene Thermocycler, tube control mode): 23-27 cycles for 95°C, 10 sec.; 60°C, 30 sec.; 72°C, 40 sec. The product of amplification was diluted 50-fold in water and one μl of this dilution was added to the second (nested) PCR. The reaction contained 1X Advantage KlenTaq Polymerase Mix with provided buffer (CLONTECH), 200 μM dNTPs, 0.2 μM of the second gene-specific primer and 0.1 μM of TS primer (SEQ ID No. 2) in a total volume of 20 μl. The cycling profile was (Hybaid OmniGene Thermocycler, tube control mode): 12 cycles for 95°C, 10 sec.; 60°C, 30 sec.; 72°C, 40 sec. The product of amplification was then cloned into pAtlas vector (CLONTECH) according to the manufacturer's protocol.

TABLE 5

Gene-Specific Primers Used for 5'-RACE

Species	First Primer	Second (Nested) Primer
Anemonia majano	5'-GAAATAGTCAGGCATACTGGT (SEQ ID No. 20)	5'-GTCAGGCATAC TGGTAGGAT (SEQ ID No. 21)
Clavularia sp.	5'-CTTGAAATAGTCTGCTATATC (SEQ ID No. 22)	5'-TCTGCTATATC GTCTGGGT (SEQ ID No. 23)
Zoanthus sp.	5'-GTTCTTGAAATAGTCTACTATGT (SEQ ID No. 24)	5'-GTCTACTATGTCTT GAGGAT (SEQ ID No. 25)
Discosoma sp. "red"	5'-CAAGCAAATGGCAAAGGTC (SEQ ID No. 26)	5'-CGGTATTGTGGCC TTCGTA (SEQ ID No. 27)
Discosoma striata	5'-TTGTCTTCTTCTGCACAAC (SEQ ID No. 28)	5'-CTGCACAACGG GTCCAT (SEQ ID No. 29)
Anemonia sulcata	5'-CCTCTATCTTCATTTCCTGC (SEQ ID No. 30)	5'-TATCTTCATTTCCT GCGTAC (SEQ ID No. 31)
Discosoma sp. "magenta"	5'-TTCAGCACCCCATCACGAG (SEQ ID No. 32)	5'-ACGCTCAGAGCTG GGTTCC (SEQ ID No. 33)
Discosoma sp. "green"	5'-CCCTCAGCAATCCATCACGTTC (SEQ ID No. 34)	5'-ATTATCTCAGTGGA TGGTTC (SEQ ID No. 35)

EXAMPLE 6

Expression of nFPs in *E.coli*

To prepare a DNA construct for novel fluorescent protein expression, two primers were synthesized for each cDNA: a 5'-directed "downstream" primer with the annealing site located in the 3'-UTR of the cDNA and a 3'-directed "upstream" primer corresponding to the site of translation start site (not including the first ATG codon) (Table 6). Both primers had 5'-heels coding for a site for a restriction endonuclease; in addition, the upstream primer was designed so as to allow the cloning of the PCR product into the pQE30 vector (Qiagen) in such a way that resulted in the fusion of reading frames of the vector-encoded 6xHis-tag and nFP. The PCR was performed as follows: 1 µl of the 20-fold dilution of the amplified cDNA sample was added to a mixture containing 1x Advantage KlenTaq Polymerase Mix with buffer provided by the manufacturer (CLONTECH), 200 µM dNTPs, 0.2 µM of upstream primer and 0.2 µM of downstream primer, in a final total volume of 20 µl. The cycling profile was (Hybaid OmniGene Thermocycler, tube control mode): 23-27 cycles for 95°C, 10 sec.; 60°C, 30 sec.; 72°C, 40 sec. The product of this amplification step was purified by phenol-chlorophorm extraction and ethanol precipitation and then cloned into pQE30 vector using restriction endonucleases corresponding to the primers' sequence according to standard protocols.

All plasmids were amplified in XL-1 blue *E. coli* and purified by plasmid DNA miniprep kits (CLONTECH). The recombinant clones were selected by colony color, and grown in 3 ml of LB medium (supplemented with 100 µg/ml of ampicillin) at 37°C overnight. 100 µl

of the overnight culture was transferred into 200 ml of fresh LB medium containing 100 μg/ml of ampicillin and grown at 37°C, 200 rpm up to OD_{600} 0.6-0.7. 1 mM IPTG was then added to the culture and incubation was allowed to proceed at 37°C for another 16 hours. The cells were harvested and recombinant protein, which incorporated 6x His tags on the N-terminus, was purified using TALON™ metal-affinity resin according to the manufacturer's protocol (CLONTECH).

TABLE 6

Primers Used to Obtain Full Coding Region of nFPs for Cloning into Expression Construct

Species	Upstream Primer	Downstream Primer
Anemonia majano	5' -acatggatccgctctttcaaaca agtttatc (SEQ ID No. 36) BamHI	5'-tagtactcgagcttattcgta tttcagtgaaatc (SEQ ID No. 37) XhoI
Clavularia sp.	L: 5'-acatggatccaacatttttttga gaaacg (SEQ ID No. 38) BamHI S: 5'-acatggatccaaagctctaacc accatg (SEQ ID No. 39) BamHI	5'-tagtactcgagcaacacaa accctcagacaa (SEQ ID No. 40) XhoI
Zoanthus sp.	5'- acatggatccgctcagtcaaag cacggt (SEQ ID No. 41) BamHI	5'-tagtactcgaggttggaactacat tcttatca (SEQ ID No. 42) XhoI
Discosoma sp. "red"	5'- acatggatccaggtcttccaagaat gttatc (SEQ ID No. 43) BamHI	5'-tagtactcgaggagccaagttc agcctta (SEQ ID No. 44) XhoI
Discosoma striata	5'- acatggatccagttggtccaagagtgtg (SEQ ID No. 45) BamHI	5'-tagcgagctctatcatgcctc gtcacct (SEQ ID No. 46) SacI
Anemonia sulcata	5'- acatggatccgcttccttttttaaagaagact (SEQ ID No. 47) BamHI	5'-tagtactcgagtccttgggagc ggcttg (SEQ ID No. 48) XhoI
Discosoma sp. "magenta"	5'- acatggatccagttgttccaagaatgtgat (SEQ ID No. 49) BamHI	5'-tagtactcgaggccattacg ctaatc (SEQ ID No. 50) XhoI
Discosoma sp. "green"	5'-acatggatccagtgcacttaaagaagaaatg (SEQ ID No. 51)	5'-tagtactcgagattcggtttaat gccttg (SEQ ID No. 52)

EXAMPLE 7

Novel Fluorescent Proteins and cDNAs Encoding the Proteins

Seven cDNA full-length cDNAs encoding fluorescent proteins were obtained (SEQ ID Nos. 45-51), and seven novel fluorescent proteins were produced (SEQ ID Nos. 53-59). The spectral properties of the isolated novel fluorescent proteins are shown in Table 7, and the emission and excitation spectra for the novel proteins are shown in Figures 3-11.

TABLE 7

Spectral Properties of the Isolated NFPs.

Species	NFP Name	Abs. Max. n m	Emission Maximum n m	Maximum Extinction Coeff.	Relative Quantum Yield*	Relative Brightness **
Anemonia majano	amFP486	458	486	40,000	0.3	0.43
Clavularia sp.	cFP484	456	484	35,300	0.6	0.77
Zoanthus sp.	zFP506	496	506	35,600	0.79	1.02
Zoanthus sp.	zFP538	528	538	20,200	0.52	0.38
Discosoma sp. "red"	drFP583	558	583	22,500	0.29	0.24
Discosoma striata	dsFP483	443	483	23,900	0.57	0.50
Anemonia sulcata	asFP600	572	596	56,200	<0.001	-
Discosoma sp "green"	dgFP512	502	512	20,360	0.3	0.21
Discosoma sp. "magenta"	dmFP592	573	593	21,800	0.11	0.09

*relative quantum yield was determined as compared to the quantum yield of *A. victoria* GFP.

**relative brightness is extinction coefficient multiplied by quantum yield divided by the same value for *A. victoria* GFP.

Multiple alignment of fluorescent proteins is shown in Figure 2A. The numbering is based on *Aequorea victoria* green fluorescent protein (GFP, SEQ ID No. 54). The amino acid sequences of the novel fluorescent proteins are labeled as SEQ ID Nos. 55-63. Two proteins from *Zoanthus* and four from *Discosoma* are compared between each other: residues identical to the corresponding ones in the first protein of the series are represented by dashes. Introduced gaps are represented by dots. In the sequence of *A. victoria* GFP, the stretches forming β-sheets are underlined; the residues whose side chains form the interior of the β-can are shaded. Figure 2B shows the N-terminal part of cFP484, which has no homology with the other proteins. The putative signal peptide is underlined.

The following references were cited herein.

1. Ormo et al., (1996) Science 273: 1392-1395.
2. Yang, F., et al., (1996) Nature Biotech 14: 1246-1251.
3. Cormack, et al., (1996) Gene 173, 33-38.
4. Haas, et al., (1996) Current Biology 6, 315-324.
5. Yang, et al., (1996) Nucleic Acids Research 24, 4592-4593.
6. Ghoda, et al.. (1990) J. Biol. Chem. 265: 11823-11826.
7. Prasher D.C. et al. (1992) Gene 111:229-33.
8. Kain et al. (1995) Biotechniques 19(4):650-55.
9. Chomczynski P., et al., (1987) Anal. Biochem. 162, 156-159.
10. Frohman et al., (1998) PNAS USA, 85, 8998-9002.

Any patents or publications mentioned in this specification are indicative of the levels of those skilled in the art to which the invention pertains. These patents and publications are herein incorporated by reference to the same extent as if each individual publication was specifically and individually indicated to be incorporated by reference.

One skilled in the art will readily appreciate that the present invention is well adapted to carry out the objects and obtain the ends and advantages mentioned, as well as those inherent therein. The present examples along with the methods, procedures, treatments, molecules, and specific compounds described herein are presently representative of preferred embodiments, are exemplary, and are not intended as limitations on the scope of the invention. Changes therein and other uses will occur to those skilled in the art which are encompassed within the spirit of the invention as defined by the scope of the claims.

WHAT IS CLAIMED IS:

1. A method of identifying a DNA sequence encoding a fluorescent protein, comprising the step of:

screening for an existence of a nucleic acid sequence in a sample, wherein said nucleic acid sequence encodes a peptide having a sequence selected from the group consisting of SEQ ID Nos. 3, 5, 8, 11, 12 and 14, and wherein the existence of said nucleic acid sequence identifies the DNA sequence encoding the fluorescent protein.

2. A method of identifying a DNA sequence encoding a fluorescent protein, comprising the step of:

screening for an existence of a nucleic acid sequence in a sample, wherein said nucleic acid sequence hybridizes to a primer selected from the group consisting of SEQ ID Nos. 4, 6, 7, 9, 10, 13, 15 and 16, and wherein the existence of said nucleic acid sequence identifies the DNA sequence encoding the fluorescent protein.

3. A method of analyzing a fluorescent protein in a cell, comprising the steps of:

a) expressing a nucleic acid sequence encoding a fluorescent protein in said cell, wherein said protein having an amino acid sequence selected from the group consisting of SEQ ID Nos. 55-63; and

b) measuring a fluorescence signal from said protein.

4. The method of claim 3, further comprising the step of:

sorting said cell according to said signal.

5. The method of claim 4, wherein said step of sorting comprises sorting said cell by fluorescence activated cell sorting.

6. The method of claim 3, wherein said nucleic acid sequence comprises a gene of interest encoding a protein of interest fused to said fluorescent protein, wherein said protein of interest is distinct from said fluorescent protein.

7. The method of claim 6, wherein the fluorescence signal indicates a presence of said gene of interest in said cell.

8. The method of claim 7, wherein said cell further comprises a protein of interest fused to said fluorescent protein.

9. The method of claim 8, further comprising the step of:

identifying an intracellular location of said fluorescent protein, thereby identifying an intracellular location of said protein of interest.

10. An isolated and purified fluorescent protein selected from the group consisting of amFP486, cFP484, zFP506, zFP538, dsFP483, drFP583, asFP600, dgFP512 and dmFP592.

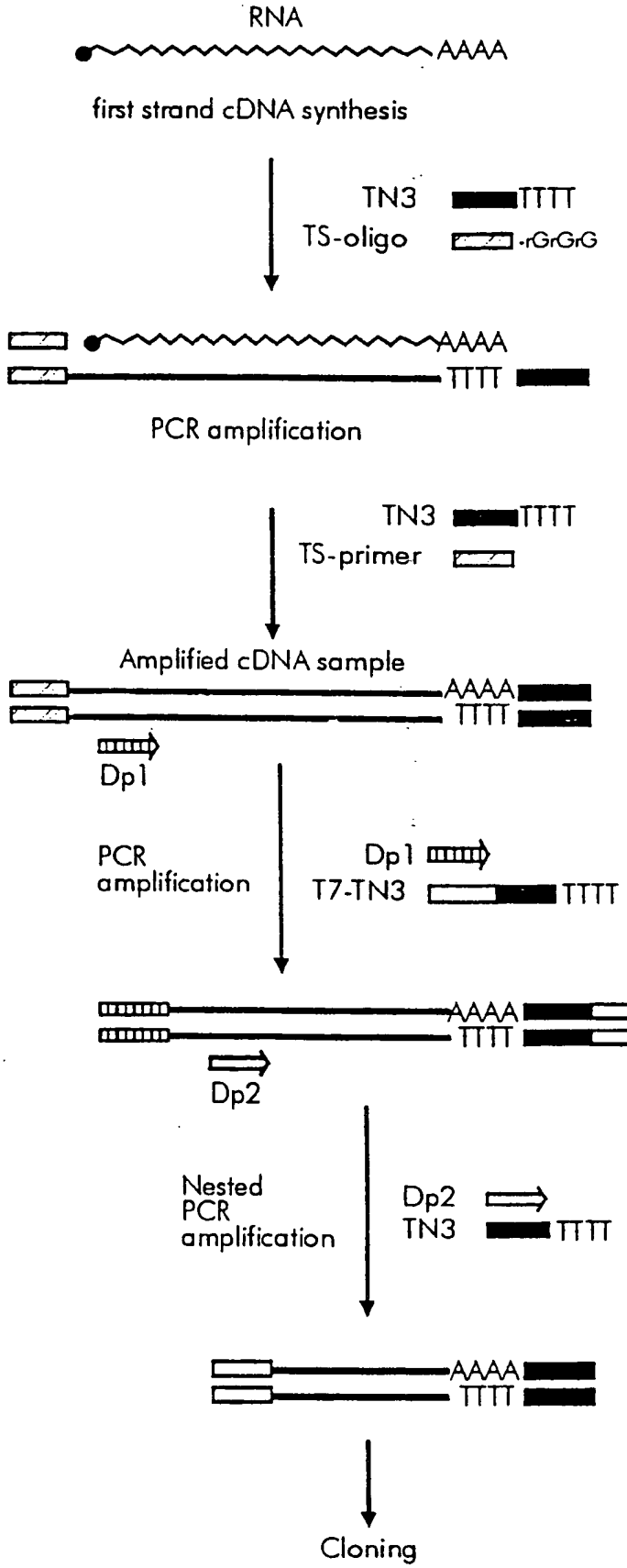

FIG. 1

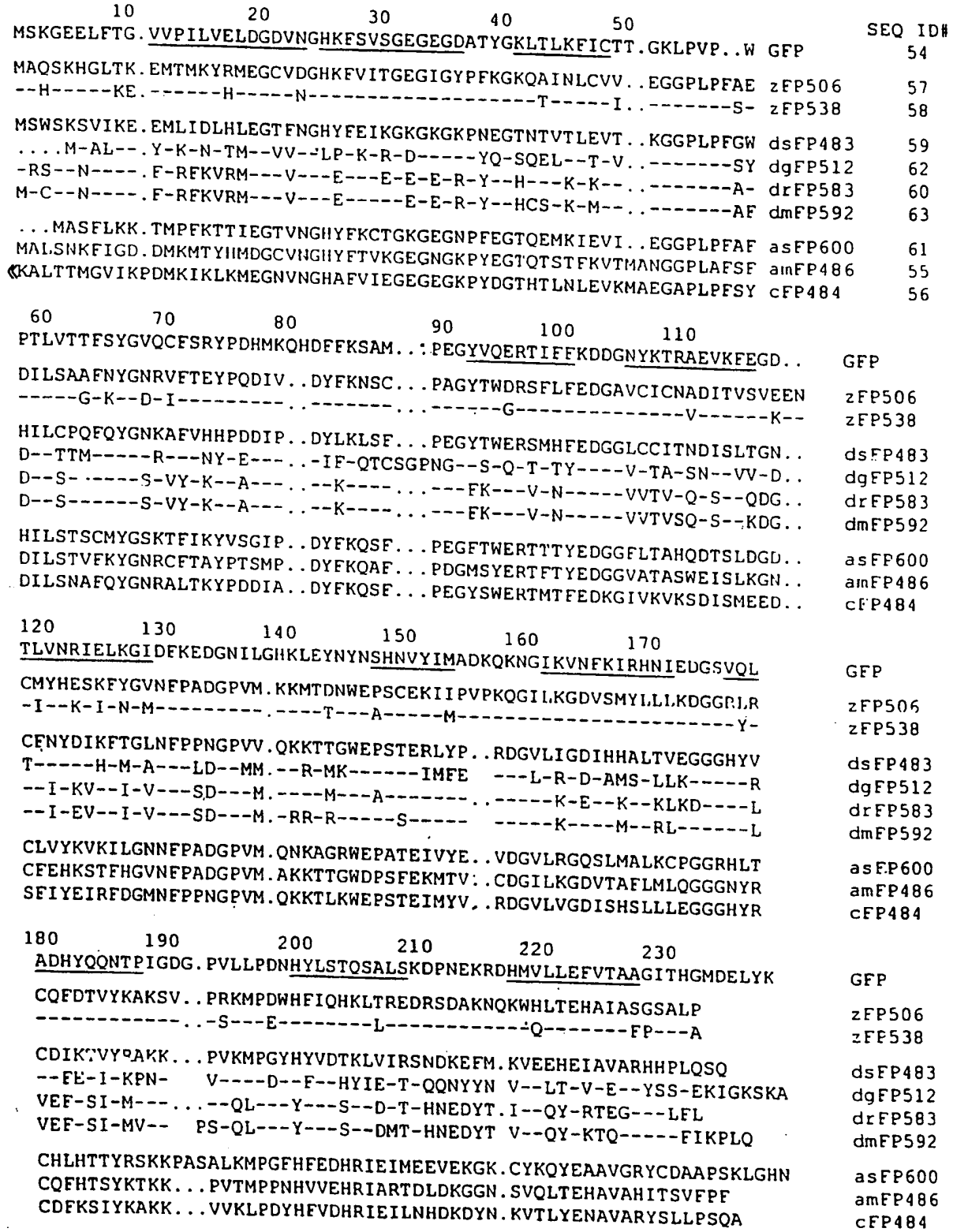

FIG. 2A

FIG. 2B

SUBSTITUTE SHEET (RULE 26)

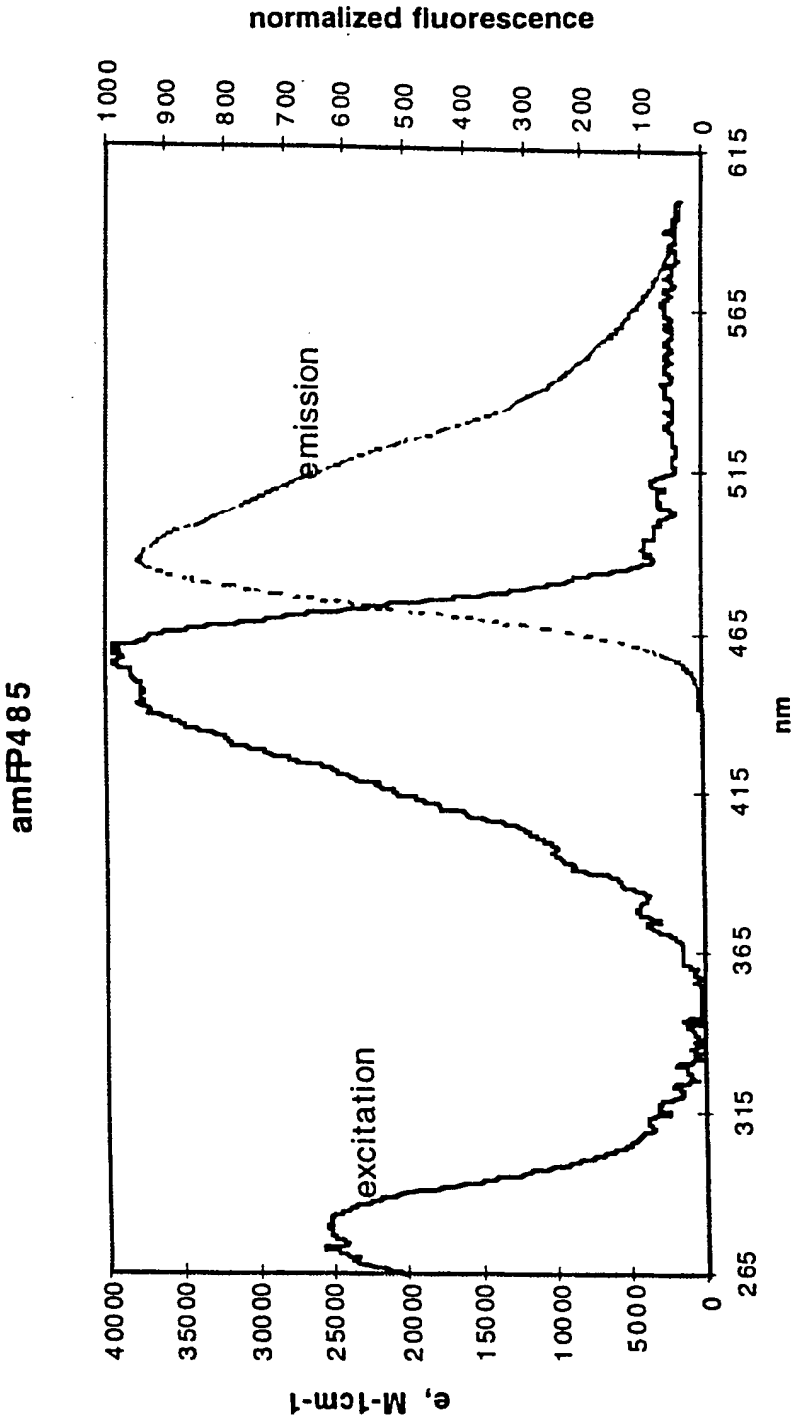

FIG. 3

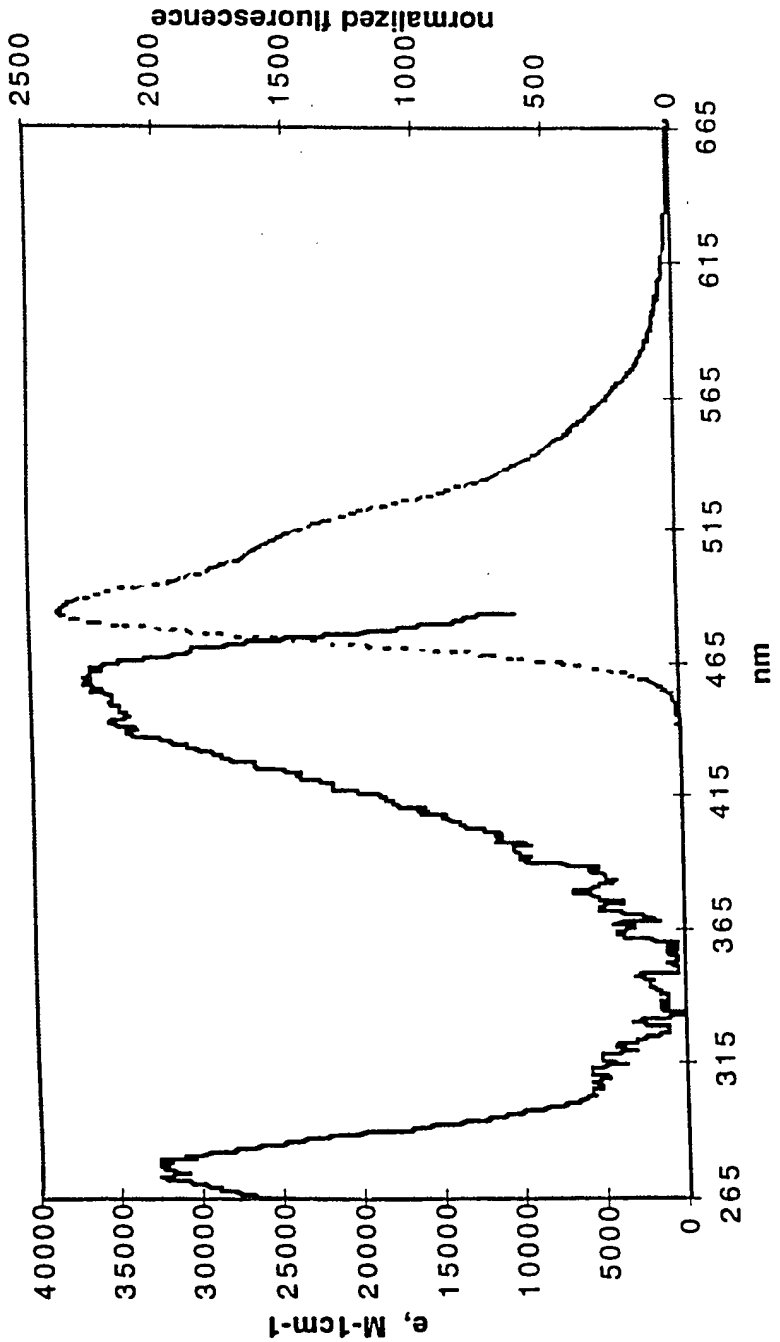

FIG. 4

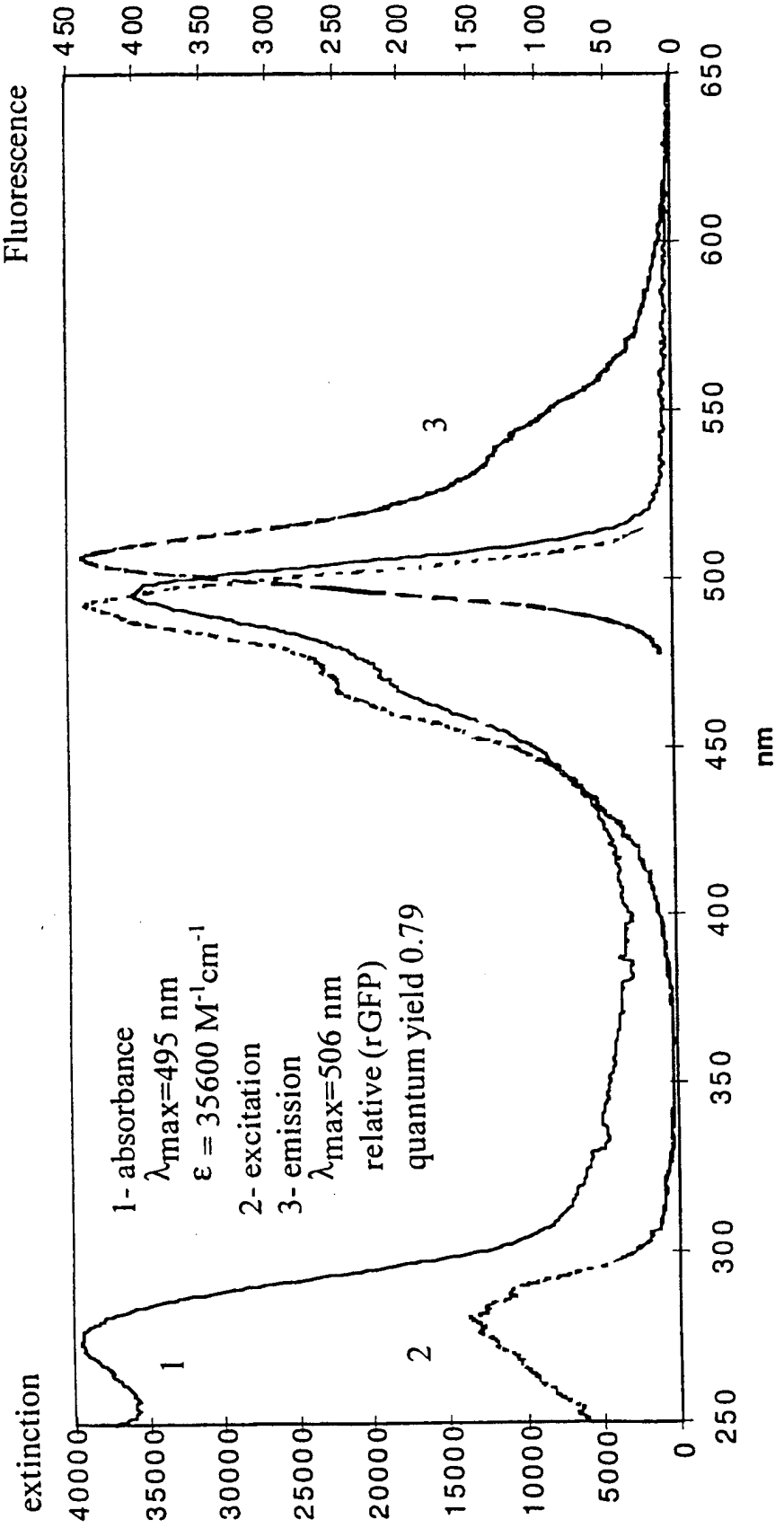

FIG. 5

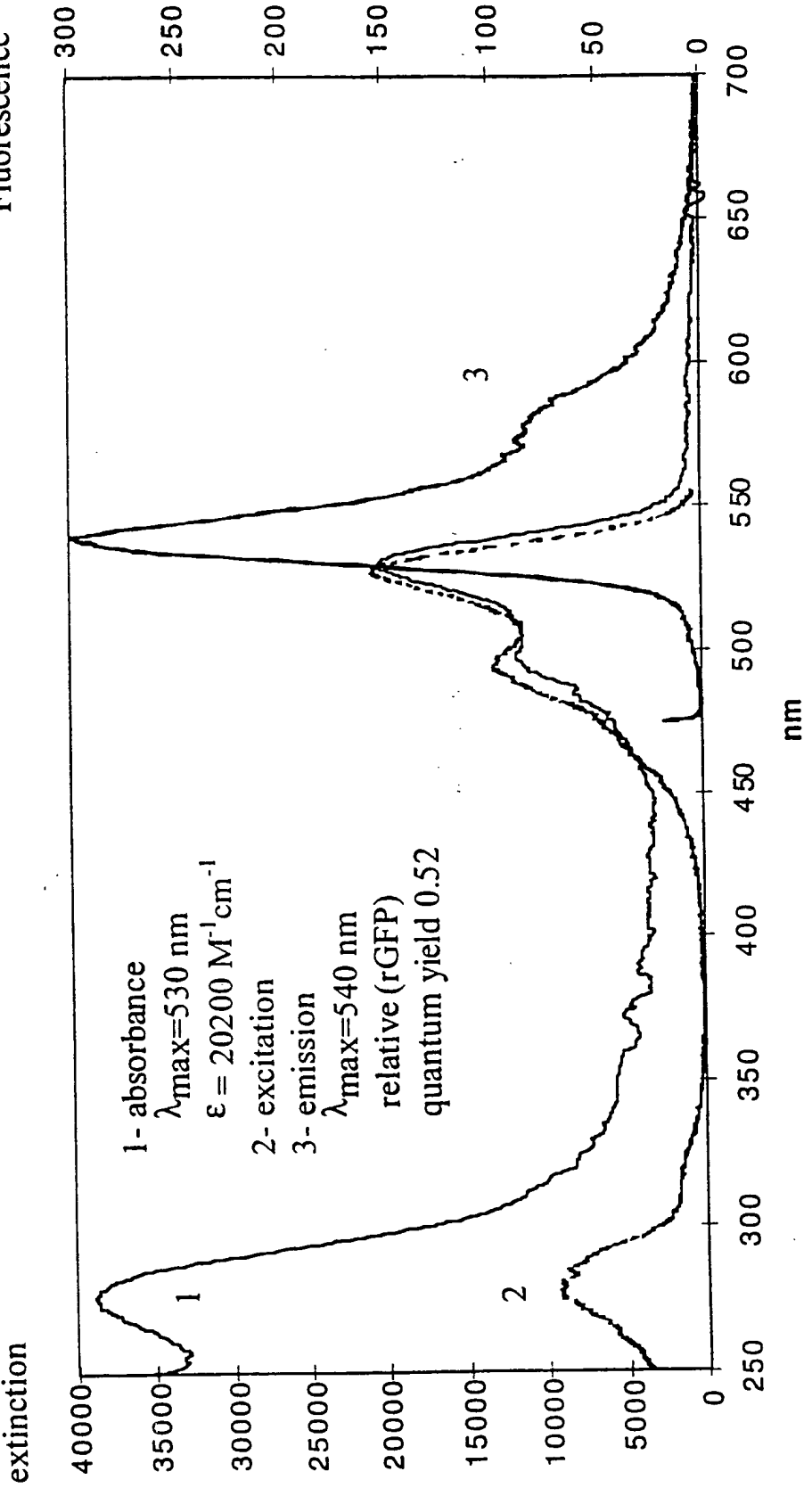

FIG. 6

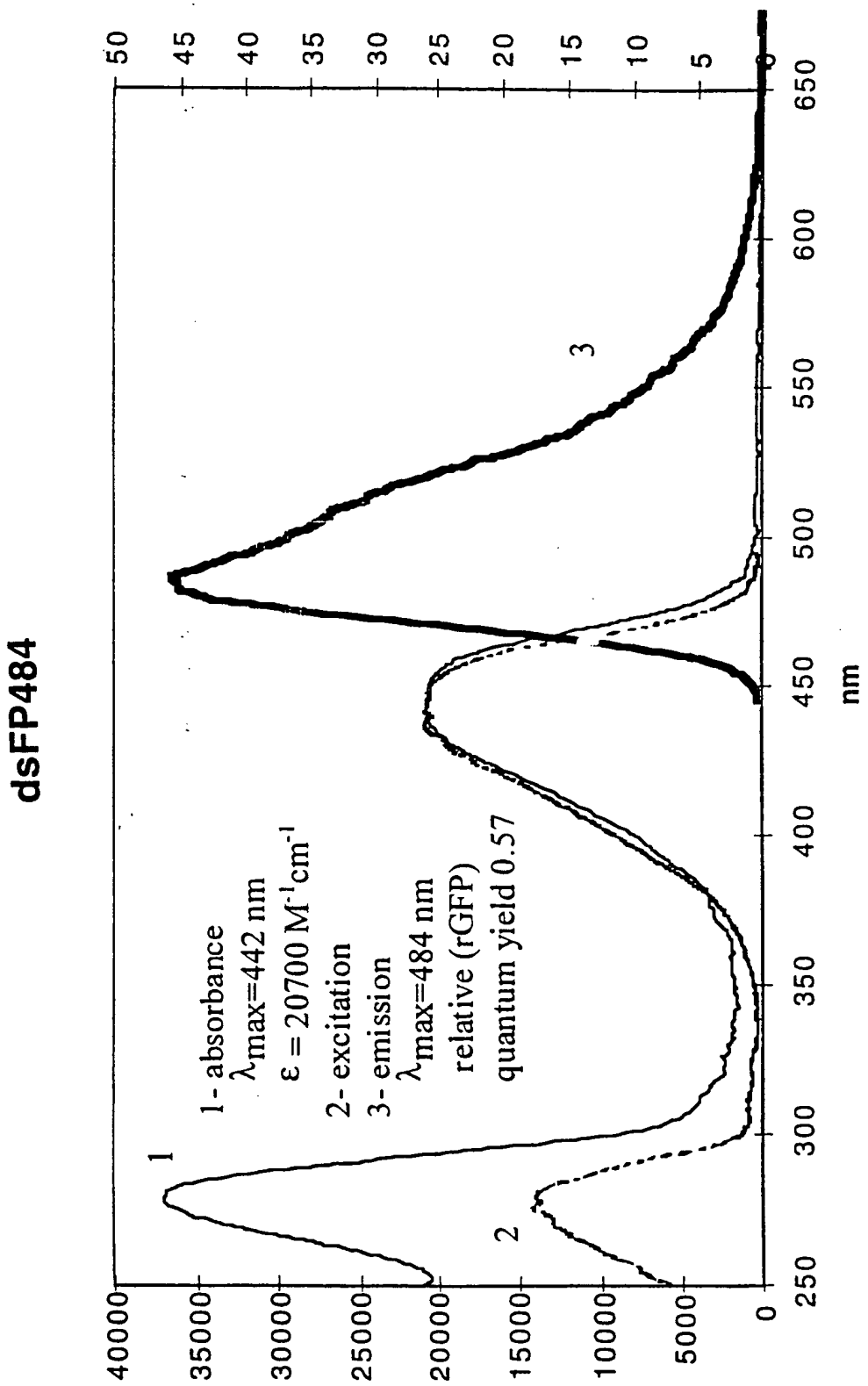

FIG. 7

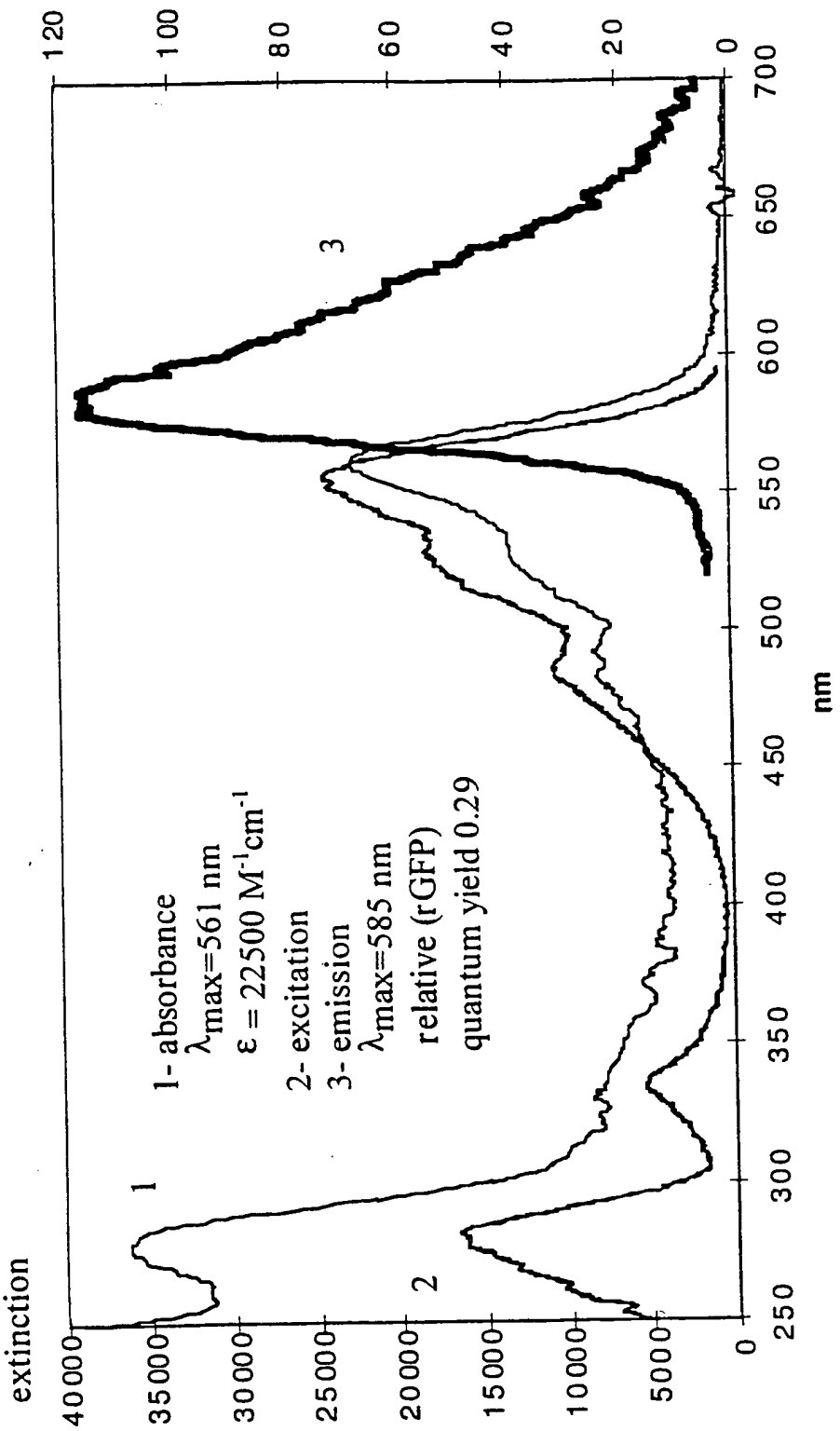

FIG. 8

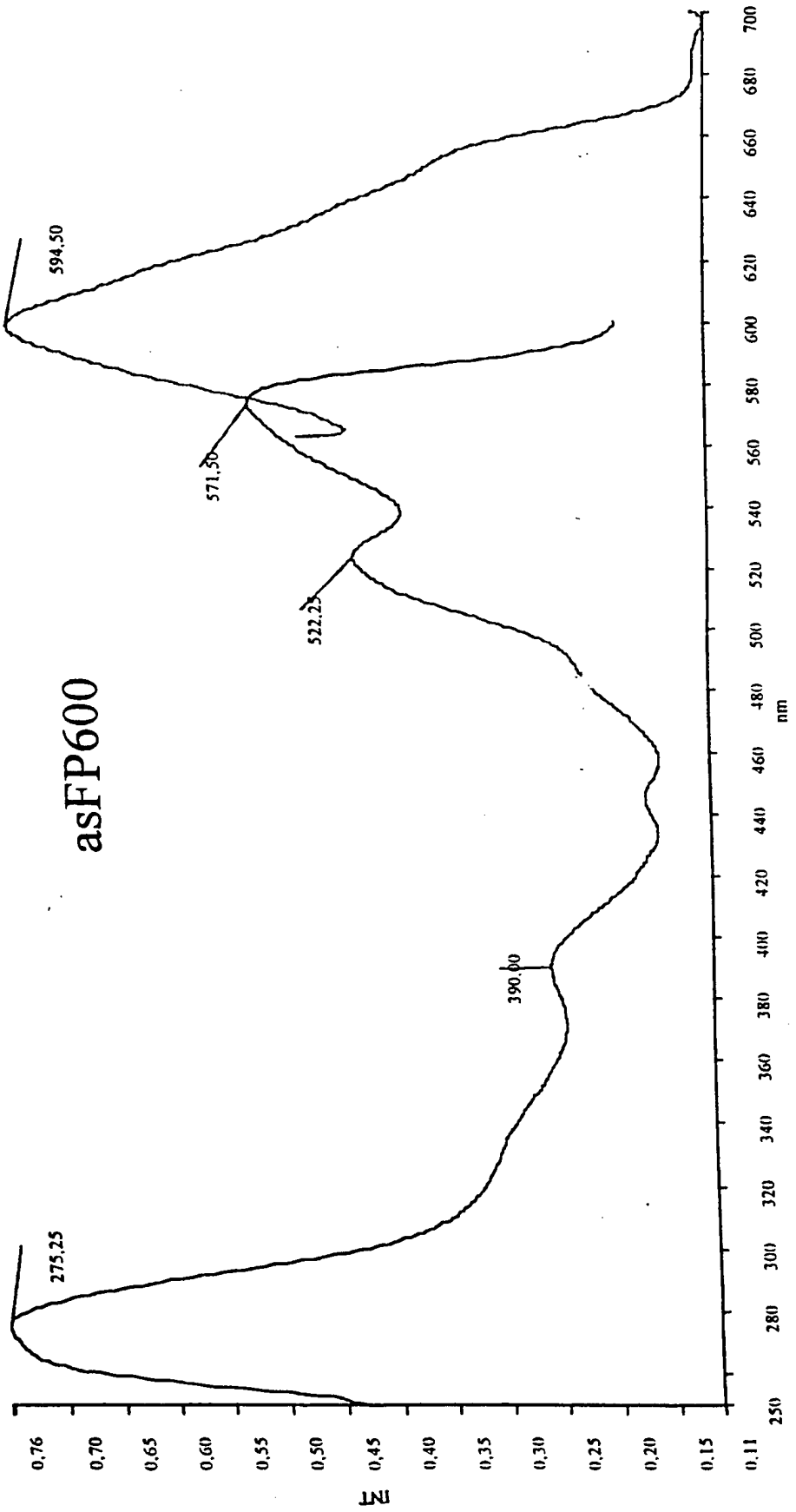

FIG. 9

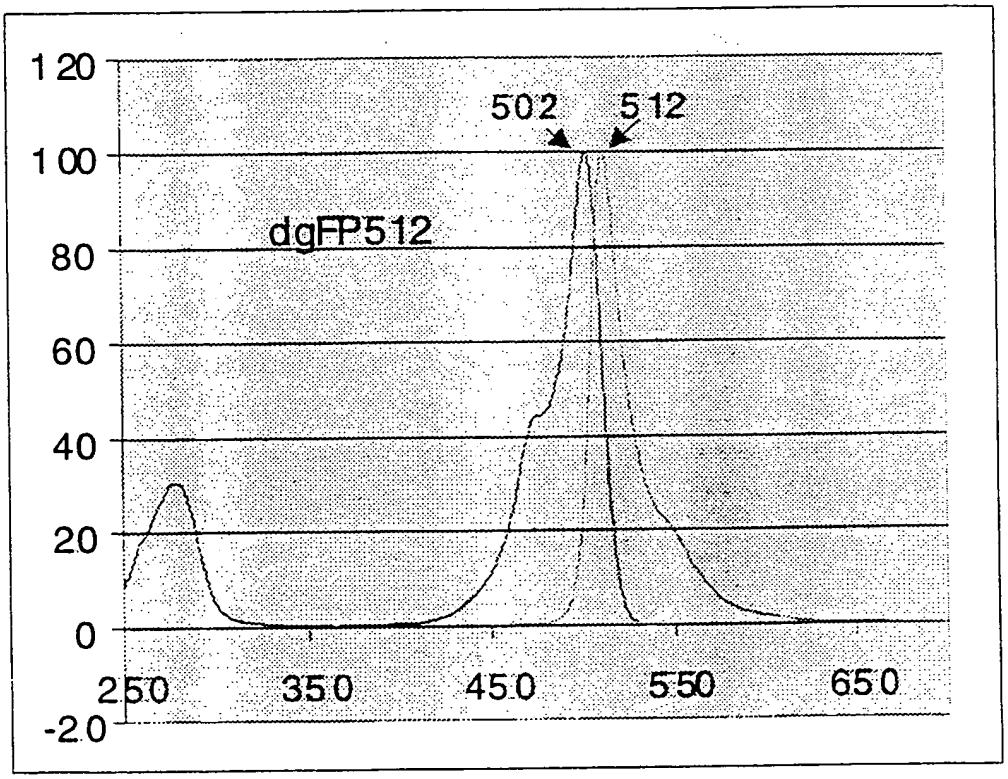

Fig. 10

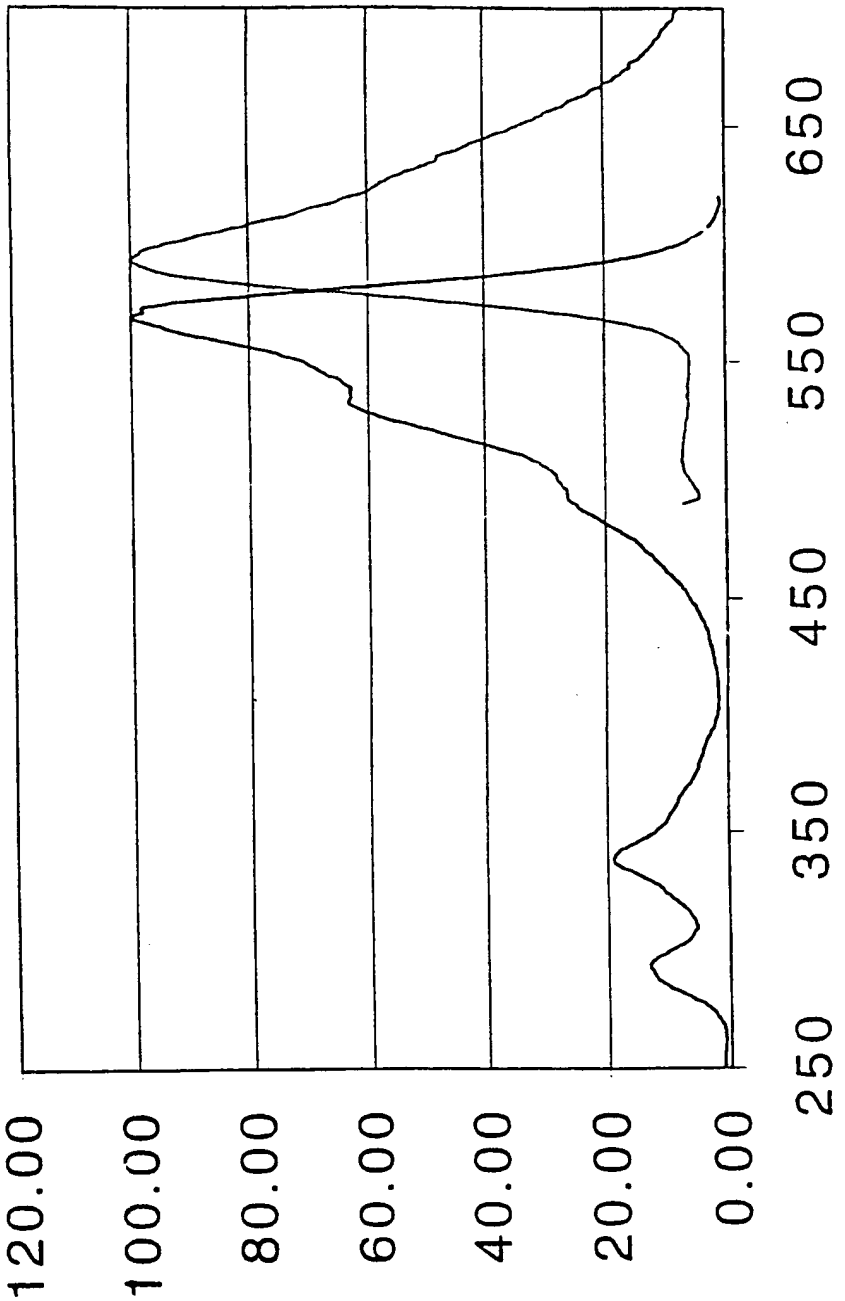

FIG. 11

SEQUENCE LISTING

<110> Lukyanov, Sergey A.
Labas, Yulii A.
Matz, Mikhail V.
Fradkov, Arcady F.

<120> Fluorescent proteins from non-bioluminescent species of Class Anthozoa, genes encoding such proteins and uses thereof

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<210> 8
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<400> 22

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<210> 23
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<400> 23

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<400> 26

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<210> 29
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<210> 30
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<210> 32
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<400> 32

ttcagcaccc catcacgag 19

<210> 33
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<400> 33

acgctcagag ctgggttcc 19

<210> 34
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<400> 34

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<210> 35
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<400> 36

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<400> 37

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of nFPs from *Clavularia sp.*

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<210> 39
<211> 28
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<210> 40
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<400> 40

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<400> 41

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<400> 42

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<400> 43

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<210> 45
<211> 28
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<220>
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<223> upstream primer used to obtain full coding region of nFPs from *Discosoma striata*
<400> 45

acatggatcc agttggtcca agagtgtg 28

<210> 46
<211> 28
<212> DNA
<213> artificial sequence
<220>
<221> primer_bind
<223> downstream primer used to obtain full coding region of nFPs from *Discosoma striata*
<400> 46

tagcgagctc tatcatgcct cgtcacct 28

<210> 47
<211> 31
<212> DNA
<213> artificial sequence
<220>
<221> primer_bind
<223> upstream primer used to obtain full coding region of nFPs from *Anemonia sulcata*
<400> 47

acatggatcc gcttcctttt taaagaagac t 31

<210> 48
<211> 28
<212> DNA
<213> artificial sequence
<220>
<221> primer_bind
<223> downstream primer used to obtain full coding

region of nFPs from *Anemonia sulcata*

<400> 48

tagtactcga gtccttggga gcggcttg 28

<210> 49
<211> 30
<212> DNA
<213> artificial sequence
<220>
<221> primer_bind
<223> upstream primer used to obtain full coding region of nFPs from *Discosoma sp. "magenta"*

<400> 49

acatggatcc agttgttcca agaatgtgat 30

<210> 50
<211> 26
<212> DNA
<213> artificial sequence
<220>
<221> primer_bind
<223> downstream primer used to obtain full coding region of nFPs from *Discosoma sp. "magenta"*

<400> 50

tagtactcga ggccattacg ctaatc 26

<210> 51
<211> 31
<212> DNA
<213> artificial sequence
<220>
<221> primer_bind
<223> upstream primer used to obtain full coding region of nFPs from *Discosoma sp. "green"*

<400> 51

acatggatcc agtgcactta aagaagaaat g 31

<210> 52
<211> 29
<212> DNA
<213> artificial sequence
<220>
<221> primer_bind
<223> downstream primer used to obtain full coding region of nFPs from *Discosoma sp. "green"*
<400> 52

tagtactcga gattcggttt aatgccttg 29

<210> 53
<211> 33
<212> DNA
<213> artificial sequence
<220>
<221> primer_bind
<223> TS-oligo used in cDNA synthesis and RACE
<400> 53

aagcagtggt atcaacgcag agtacgcrgr grg 33

<210> 54
<211> 238
<212> PRT
<213> *Aequorea victoria*
<220>
<223> amino acid sequence of GFP
<400> 54

Met Ser Lys Gly Glu Glu Leu Phe Thr Gly Val Val Pro Ile Leu
5 10 15
Val Glu Leu Asp Gly Asp Val Asn Gly His Lys Phe Ser Val Ser
20 25 30
Gly Glu Gly Glu Gly Asp Ala Thr Tyr Gly Lys Leu Thr Leu Lys
35 40 45
Phe Ile Cys Thr Thr Gly Lys Leu Pro Val Pro Trp Pro Thr Leu
50 55 60

Val Thr Thr Phe Ser Tyr Gly Val Gln Cys Phe Ser Arg Tyr Pro
65 70 75
Asp His Met Lys Gln His Asp Phe Phe Lys Ser Ala Met Pro Glu
80 85 90
Gly Tyr Val Gln Glu Arg Thr Ile Phe Phe Lys Asp Asp Gly Asn
95 100 105
Tyr Lys Thr Arg Ala Glu Val Lys Phe Glu Gly Asp Thr Leu Val
110 115 120
Asn Arg Ile Glu Leu Lys Gly Ile Asp Phe Lys Glu Asp Gly Asn
125 130 135
Ile Leu Gly His Lys Leu Glu Tyr Asn Tyr Asn Ser His Asn Val
140 145 150
Tyr Ile Met Ala Asp Lys Gln Lys Asn Gly Ile Lys Val Asn Phe
155 160 165
Lys Ile Arg His Asn Ile Glu Asp Gly Ser Val Gln Leu Ala Asp
170 175 180
His Tyr Gln Gln Asn Thr Pro Ile Gly Asp Gly Pro Val Leu Leu
185 190 195
Pro Asp Asn His Tyr Leu Ser Thr Gln Ser Ala Leu Ser Lys Asp
200 205 210
Pro Asn Glu Lys Arg Asp His Met Val Leu Leu Glu Phe Val Thr
215 220 225
Ala Ala Gly Ile Thr His Gly Met Asp Glu Leu Tyr Lys
230 235

<210> 55
<211> 229
<212> PRT
<213> *Anemonia majano*
<220>
<223> amino acid sequence of amFP486
<400> 55

Met Ala Leu Ser Asn Lys Phe Ile Gly Asp Asp Met Lys Met Thr
5 10 15
Tyr His Met Asp Gly Cys Val Asn Gly His Tyr Phe Thr Val Lys
20 25 30
Gly Glu Gly Asn Gly Lys Pro Tyr Glu Gly Thr Gln Thr Ser Thr
35 40 45

Phe Lys Val Thr Met Ala Asn Gly Gly Pro Leu Ala Phe Ser Phe
50 55 60
Asp Ile Leu Ser Thr Val Phe Lys Tyr Gly Asn Arg Cys Phe Thr
65 70 75
Ala Tyr Pro Thr Ser Met Pro Asp Tyr Phe Lys Gln Ala Phe Pro
80 85 90
Asp Gly Met Ser Tyr Glu Arg Thr Phe Thr Tyr Glu Asp Gly Gly
95 100 105
Val Ala Thr Ala Ser Trp Glu Ile Ser Leu Lys Gly Asn Cys Phe
110 115 120
Glu His Lys Ser Thr Phe His Gly Val Asn Phe Pro Ala Asp Gly
125 130 135
Pro Val Met Ala Lys Lys Thr Thr Gly Trp Asp Pro Ser Phe Glu
140 145 150
Lys Met Thr Val Cys Asp Gly Ile Leu Lys Gly Asp Val Thr Ala
155 160 165
Phe Leu Met Leu Gln Gly Gly Gly Asn Tyr Arg Cys Gln Phe His
170 175 180
Thr Ser Tyr Lys Thr Lys Lys Pro Val Thr Met Pro Pro Asn His
185 190 195
Val Val Glu His Arg Ile Ala Arg Thr Asp Leu Asp Lys Gly Gly
200 205 210
Asn Ser Val Gln Leu Thr Glu His Ala Val Ala His Ile Thr Ser
215 220 225
Val Val Pro Phe

<210> 56
<211> 266
<212> PRT
<213> *Clavularia sp.*
<220>
<223> amino acid sequence of cFP484
<400> 56
Met Lys Cys Lys Phe Val Phe Cys Leu Ser Phe Leu Val Leu Ala
5 10 15
Ile Thr Asn Ala Asn Ile Phe Leu Arg Asn Glu Ala Asp Phe Glu

20 25 30
Glu Lys Thr Phe Arg Ile Pro Lys Ala Leu Thr Thr Met Gly Val
35 40 45
Ile Lys Pro Asp Met Lys Ile Lys Leu Lys Met Glu Gly Asn Val
50 55 60
Asn Gly His Ala Phe Val Ile Glu Gly Glu Gly Glu Gly Lys Pro
65 70 75
Tyr Asp Gly Thr His Thr Leu Asn Leu Glu Val Lys Glu Gly Ala
80 85 90
Pro Leu Pro Phe Ser Tyr Asp Ile Leu Ser Asn Ala Phe Gln Tyr
95 100 105
Gly Asn Arg Ala Leu Thr Lys Tyr Pro Asp Asp Ile Ala Asp Tyr
110 115 120
Phe Lys Gln Ser Phe Pro Glu Gly Tyr Ser Trp Glu Arg Thr Met
125 130 135
Thr Phe Glu Asp Lys Gly Ile Val Lys Val Lys Ser Asp Ile Ser
140 145 150
Met Glu Glu Asp Ser Phe Ile Tyr Glu Ile Arg Phe Asp Gly Met
155 160 165
Asp Phe Pro Pro Asn Gly Pro Val Met Gln Lys Lys Thr Leu Lys
170 175 180
Trp Glu Pro Ser Thr Glu Ile Met Tyr Val Arg Asp Gly Val Leu
185 190 195
Val Gly Asp Ile Ser His Ser Leu Leu Leu Glu Gly Gly Gly His
200 205 210
Tyr Arg Cys Asp Phe Lys Ser Ile Tyr Lys Ala Lys Lys Val Val
215 220 225
Lys Leu Pro Asp Tyr His Phe Val Asp His Arg Ile Glu Ile Leu
230 235 240
Asn His Asp Lys Asp Tyr Asn Lys Val Thr Leu Tyr Glu Asn Ala
245 250 255
Val Ala Arg Tyr Ser Leu Leu Pro Ser Gln Ala
260 265

<210> 57
<211> 230
<212> PRT

<213> *Zoanthus sp.*

<220>

<223> amino acid sequence of zFP506

<400> 57

Ala Gln Ser Lys His Gly Leu Thr Lys Glu Met Thr Met Lys Tyr
5 10 15

Arg Met Glu Gly Cys Val Asp Gly His Lys Phe Val Ile Thr Gly
20 25 30

Glu Gly Ile Gly Tyr Pro Phe Lys Gly Lys Gln Ala Ile Asn Leu
35 40 45

Cys Val Val Glu Gly Gly Pro Leu Pro Phe Ala Glu Asp Ile Leu
50 55 60

Ser Ala Ala Phe Asn Tyr Gly Asn Arg Val Phe Thr Glu Tyr Pro
65 70 75

Gln Asp Ile Val Asp Tyr Phe Lys Asn Ser Cys Pro Ala Gly Tyr
80 85 90

Thr Trp Asp Arg Ser Phe Leu Phe Glu Asp Gly Ala Val Cys Ile
95 100 105

Cys Asn Ala Asp Ile Thr Val Ser Val Glu Glu Asn Cys Met Tyr
110 115 120

His Glu Ser Lys Phe Tyr Gly Val Asn Phe Pro Ala Asp Gly Pro
125 130 135

Val Met Lys Lys Met Thr Asp Asn Trp Glu Pro Ser Cys Glu Lys
140 145 150

Ile Ile Pro Val Pro Lys Gln Gly Ile Leu Lys Gly Asp Val Ser
155 160 165

Met Tyr Leu Leu Leu Lys Asp Gly Gly Arg Leu Arg Cys Gln Phe
170 175 180

Asp Thr Val Tyr Lys Ala Lys Ser Val Pro Arg Lys Met Pro Asp
185 190 195

Trp His Phe Ile Gln His Lys Leu Thr Arg Glu Asp Arg Ser Asp
200 205 210

Ala Lys Asn Gln Lys Trp His Leu Thr Glu His Ala Ile Ala Ser
215 220 225

Gly Ser Ala Leu Pro
230

<210> 58
<211> 230
<212> PRT
<213> *Zoanthus sp.*
<220>
<223> amino acid sequence of zFP538
<400> 58

Met Ala His Ser Lys His Gly Leu Lys Glu Glu Met Thr Met Lys
5 10 15
Tyr His Met Glu Gly Cys Val Asn Gly His Lys Phe Val Ile Thr
20 25 30
Gly Glu Gly Ile Gly Tyr Pro Phe Lys Gly Lys Gln Thr Ile Asn
35 40 45
Leu Cys Val Ile Glu Gly Gly Pro Leu Pro Phe Ser Glu Asp Ile
50 55 60
Leu Ser Ala Gly Phe Lys Tyr Gly Asp Arg Ile Phe Thr Glu Tyr
65 70 75
Pro Gln Asp Ile Val Asp Tyr Phe Lys Asn Ser Cys Pro Ala Gly
80 85 90
Tyr Thr Trp Gly Ser Phe Leu Phe Glu Asp Gly Ala Val Cys Ile
95 100 105
Cys Asn Val Asp Ile Thr Val Ser Val Lys Glu Asn Cys Ile Tyr
110 115 120
His Lys Ser Ile Phe Asn Gly Met Asn Phe Pro Ala Asp Gly Pro
125 130 135
Val Met Lys Lys Met Thr Thr Asn Trp Glu Ala Ser Cys Glu Lys
140 145 150
Ile Met Pro Val Pro Lys Gln Gly Ile Leu Lys Gly Asp Val Ser
155 160 165
Met Tyr Leu Leu Leu Lys Asp Gly Gly Arg Tyr Arg Cys Gln Phe
170 175 180
Asp Thr Val Tyr Lys Ala Lys Ser Val Pro Ser Lys Met Pro Glu
185 190 195
Trp His Phe Ile Gln His Lys Leu Leu Arg Glu Asp Arg Ser Asp
200 205 210
Ala Lys Asn Gln Lys Trp Gln Leu Thr Glu His Ala Ile Ala Phe
215 220 225

Pro Ser Ala Leu Ala
230

<210> 59
<211> 232
<212> PRT
<213> *Discosoma striata*
<220>
<223> amino acid sequence of dsFP483
<400> 59

Met Ser Cys Ser Lys Ser Val Ile Lys Glu Glu Met Leu Ile Asp
5 10 15
Leu His Leu Glu Gly Thr Phe Asn Gly His Tyr Phe Glu Ile Lys
20 25 30
Gly Lys Gly Lys Gly Gln Pro Asn Glu Gly Thr Asn Thr Val Thr
35 40 45
Leu Glu Val Thr Lys Gly Gly Pro Leu Pro Phe Gly Trp His Ile
50 55 60
Leu Cys Pro Gln Phe Gln Tyr Gly Asn Lys Ala Phe Val His His
65 70 75
Pro Asp Asn Ile His Asp Tyr Leu Lys Leu Ser Phe Pro Glu Gly
80 85 90
Tyr Thr Trp Glu Arg Ser Met His Phe Glu Asp Gly Gly Leu Cys
95 100 105
Cys Ile Thr Asn Asp Ile Ser Leu Thr Gly Asn Cys Phe Tyr Tyr
110 115 120
Asp Ile Lys Phe Thr Gly Leu Asn Phe Pro Pro Asn Gly Pro Val
125 130 135
Val Gln Lys Lys Thr Thr Gly Trp Glu Pro Ser Thr Glu Arg Leu
140 145 150
Tyr Pro Arg Asp Gly Val Leu Ile Gly Asp Ile His His Ala Leu
155 160 165
Thr Val Glu Gly Gly Gly His Tyr Ala Cys Asp Ile Lys Thr Val
170 175 180
Tyr Arg Ala Lys Lys Ala Ala Leu Lys Met Pro Gly Tyr His Tyr
185 190 195

Val Asp Thr Lys Leu Val Ile Trp Asn Asn Asp Lys Glu Phe Met
200 205 210
Lys Val Glu Glu His Glu Ile Ala Val Ala Arg His His Pro Phe
215 220 225
Tyr Glu Pro Lys Lys Asp Lys
230

<210> 60
<211> 225
<212> PRT
<213> *Discosoma sp.* "red"
<220>
<223> amino acid sequence of drFP583
<400> 60
Met Arg Ser Ser Lys Asn Val Ile Lys Glu Phe Met Arg Phe Lys
5 10 15
Val Arg Met Glu Gly Thr Val Asn Gly His Glu Phe Glu Ile Glu
20 25 30
Gly Glu Gly Glu Gly Arg Pro Tyr Glu Gly His Asn Thr Val Lys
35 40 45
Leu Lys Val Thr Lys Gly Gly Pro Leu Pro Phe Ala Trp Asp Ile
50 55 60
Leu Ser Pro Gln Phe Gln Tyr Gly Ser Lys Val Tyr Val Lys His
65 70 75
Pro Ala Asp Ile Pro Asp Tyr Lys Lys Leu Ser Phe Pro Glu Gly
80 85 90
Phe Lys Trp Glu Arg Val Met Asn Phe Glu Asp Gly Gly Val Val
95 100 105
Thr Val Thr Gln Asp Ser Ser Leu Gln Asp Gly Cys Phe Ile Tyr
110 115 120
Lys Val Lys Phe Ile Gly Val Asn Phe Pro Ser Asp Gly Pro Val
125 130 135
Met Gln Lys Lys Thr Met Gly Trp Glu Ala Ser Thr Glu Arg Leu
140 145 150
Tyr Pro Arg Asp Gly Val Leu Lys Gly Glu Ile His Lys Ala Leu
155 160 165
Lys Leu Lys Asp Gly Gly His Tyr Leu Val Glu Phe Lys Ser Ile
170 175 180

Tyr Met Ala Lys Lys Pro Val Gln Leu Pro Gly Tyr Tyr Tyr Val
185 190 195
Asp Ser Lys Leu Asp Ile Thr Ser His Asn Glu Asp Tyr Thr Ile
200 205 210
Val Glu Gln Tyr Glu Arg Thr Glu Gly Arg His His Leu Phe Leu
215 220 225

<210> 61
<211> 232
<212> PRT
<213> *Anemonia sulcata*
<220>
<223> amino acid sequence of asFP600
<400> 61

Met Ala Ser Phe Leu Lys Lys Thr Met Pro Phe Lys Thr Thr Ile
5 10 15
Glu Gly Thr Val Asn Gly His Tyr Phe Lys Cys Thr Gly Lys Gly
20 25 30
Glu Gly Asn Pro Phe Glu Gly Thr Gln Glu Met Lys Ile Glu Val
35 40 45
Ile Glu Gly Gly Pro Leu Pro Phe Ala Phe His Ile Leu Ser Thr
50 55 60
Ser Cys Met Tyr Gly Ser Lys Thr Phe Ile Lys Tyr Val Ser Gly
65 70 75
Ile Pro Asp Tyr Phe Lys Gln Ser Phe Pro Glu Gly Phe Thr Trp
80 85 90
Glu Arg Thr Thr Thr Tyr Glu Asp Gly Gly Phe Leu Thr Ala His
95 100 105
Gln Asp Thr Ser Leu Asp Gly Asp Cys Leu Val Tyr Lys Val Lys
110 115 120
Ile Leu Gly Asn Asn Phe Pro Ala Asp Gly Pro Val Met Gln Asn
125 130 135
Lys Ala Gly Arg Trp Glu Pro Ala Thr Glu Ile Val Tyr Glu Val
140 145 150
Asp Gly Val Leu Arg Gly Gln Ser Leu Met Ala Leu Lys Cys Pro
155 160 165
Gly Gly Arg His Leu Thr Cys His Leu His Thr Thr Tyr Arg Ser
170 175 180

Lys Lys Pro Ala Ser Ala Leu Lys Met Pro Gly Phe His Phe Glu
185 190 195
Asp His Arg Ile Glu Ile Met Glu Glu Val Glu Lys Gly Lys Cys
200 205 210
Tyr Lys Gln Tyr Glu Ala Ala Val Gly Arg Tyr Cys Asp Ala Ala
215 220 225
Pro Ser Lys Leu Gly His Asn
230

<210> 62
<211> 231
<212> PRT
<213> *Discosoma sp. "green"*
<220>
<223> amino acid sequence of dgFP512
<400> 62

Met Ser Ala Leu Lys Glu Glu Met Lys Ile Asn Leu Thr Met Glu
5 10 15
Gly Val Val Asn Gly Leu Pro Phe Lys Ile Arg Gly Asp Gly Lys
20 25 30
Gly Lys Pro Tyr Gln Gly Ser Gln Glu Leu Thr Leu Thr Val Val
35 40 45
Lys Gly Gly Pro Leu Pro Phe Ser Tyr Asp Ile Leu Thr Thr Met
50 55 60
Phe Gln Tyr Gly Asn Arg Ala Phe Val Asn Tyr Pro Glu Asp Ile
65 70 75
Pro Asp Ile Phe Lys Gln Thr Cys Ser Gly Pro Asn Gly Gly Tyr
80 85 90
Ser Trp Gln Arg Thr Met Thr Tyr Glu Asp Gly Gly Val Cys Thr
95 100 105
Ala Thr Ser Asn Ile Ser Val Val Gly Asp Thr Phe Asn Tyr Asp
110 115 120

Ile His Phe Met Gly Ala Asn Phe Pro Leu Asp Gly Pro Val Met
125 130 135

Gln Lys Arg Thr Met Lys Trp Glu Pro Ser Thr Glu Ile Met Phe
140 145 150

Glu Arg Asp Gly Met Leu Arg Gly Asp Ile Ala Met Ser Leu Leu
155 160 165

Leu Lys Gly Gly Gly His Tyr Arg Cys Asp Phe Glu Thr Ile Tyr
170 175 180

Lys Pro Asn Lys Val Val Lys Met Pro Asp Tyr His Phe Val Asp
185 190 195

His Cys Ile Glu Ile Thr Ser Gln Gln Asp Tyr Tyr Asn Val Val
200 205 210

Glu Leu Thr Glu Val Ala Glu Ala Arg Tyr Ser Ser Leu Glu Lys
215 220 225

Ile Gly Lys Ser Lys Ala
230

<210> 63
<211> 235
<212> PRT
<213> *Discosoma sp. "magenta"*
<220>
<223> amino acid sequence of dmFP592
<400> 63

Met Ser Cys Ser Lys Asn Val Ile Lys Glu Phe Met Arg Phe Lys
5 10 15

Val Arg Met Glu Gly Thr Val Asn Gly His Glu Phe Glu Ile Lys
20 25 30

Gly Glu Gly Glu Gly Arg Pro Tyr Glu Gly His Cys Ser Val Lys
35 40 45

Leu Met Val Thr Lys Gly Gly Pro Leu Pro Phe Ala Phe Asp Ile
50 55 60

Leu Ser Pro Gln Phe Gln Tyr Gly Ser Lys Val Tyr Val Lys His
65 70 75

Pro Ala Asp Ile Pro Asp Tyr Lys Lys Leu Ser Phe Pro Glu Gly
80 85 90

Phe Lys Trp Glu Arg Val Met Asn Phe Glu Asp Gly Gly Val Val
100 105 110

Thr Val Ser Gln Asp Ser Ser Leu Lys Asp Gly Cys Phe Ile Tyr
115 120 125

Glu Val Lys Phe Ile Gly Val Asn Phe Pro Ser Asp Gly Pro Val
130 135 140

Met Gln Arg Arg Thr Arg Gly Trp Glu Ala Ser Ser Glu Arg Leu
145 150 155

Tyr Pro Arg Asp Gly Val Leu Lys Gly Asp Ile His Met Ala Leu
160 165 170

Arg Leu Glu Gly Gly Gly His Tyr Leu Val Glu Phe Lys Ser Ile
175 180 185

Tyr Met Val Lys Lys Pro Ser Val Gln Leu Pro Gly Tyr Tyr Tyr
190 195 200

Val Asp Ser Lys Leu Asp Met Thr Ser His Asn Glu Asp Tyr Thr
205 210 215

Val Val Glu Gln Tyr Glu Lys Thr Gln Gly Arg His His Pro Phe
220 225 230

Ile Lys Pro Leu Gln
235

INTERNATIONAL SEARCH REPORT

International application No.
PCT/US99/29405

A. CLASSIFICATION OF SUBJECT MATTER

IPC(7) :C12Q 1/68; C07K 14/435
US CL :435/6, 69.1; 530/350
According to International Patent Classification (IPC) or to both national classification and IPC

B. FIELDS SEARCHED

Minimum documentation searched (classification system followed by classification symbols)

U.S. : 435/6, 69.1, 968; 530/350; 424/9.6, 436/172

Documentation searched other than minimum documentation to the extent that such documents are included in the fields searched

Electronic data base consulted during the international search (name of data base and, where practicable, search terms used)

Please See Extra Sheet.

C. DOCUMENTS CONSIDERED TO BE RELEVANT

Category*	Citation of document, with indication, where appropriate, of the relevant passages	Relevant to claim No.
***	The sequence diskette submitted with the description was defective; thus the references listed below were obtained solely by a WORD search, and not by a search of the SEQ ID NOs.	***
X, P	MATZ et al. Fluorescent proteins from nonbioluminescent Anthozoa species. Nature Biotechnology. October 1999, Volume 17, No. 10, pages 969-973, entire document.	1-10
X, P	DE 197 18 640 A1 (WIEDENMANN) 22 July 1999, entire document.	3-10

☐ Further documents are listed in the continuation of Box C. ☐ See patent family annex.

*	Special categories of cited documents:	"T"	later document published after the international filing date or priority date and not in conflict with the application but cited to understand the principle or theory underlying the invention
"A"	document defining the general state of the art which is not considered to be of particular relevance	"X"	document of particular relevance; the claimed invention cannot be considered novel or cannot be considered to involve an inventive step when the document is taken alone
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"L"	document which may throw doubts on priority claim(s) or which is cited to establish the publication date of another citation or other special reason (as specified)	"&"	document member of the same patent family
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"P"	document published prior to the international filing date but later than the priority date claimed		

Date of the actual completion of the international search 18 FEBRUARY 2000	Date of mailing of the international search report 02 MAR 2000
Name and mailing address of the ISA/US Commissioner of Patents and Trademarks Box PCT Washington, D.C. 20231 Facsimile No. (703) 305-3230	Authorized officer GABRIELE ELISABETH BOGAISKY Telephone No. (703) 308-0196

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